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HYDANTOIN DERIVATIVES. A NEW CLASS OF INHIBITORS OF HUMAN LEUKOCYTE ELASTASE

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Derivatives of hydantoin have been found to inactivate human leukocyte elastase irreversibly. Chymotrypsin and cathepsin G are also inhibited by these compounds.

KEY WORDS: Hydantoin derivatives; human leukocyte elastase inhibitors.

INTRODUCTION

Proteolytic enzymes appear to play a major role in the degradation of connective tissue components and have been implicated in cancer metastasis,[1] pulmonary emphysema,[2] rheumatoid arthritis,[3] neonatal and adult respiratory distress syndrome[4] and other diseases.[5] The lysosomal serine protease human leukocyte elastase (HLE) degrades lung elastin[6] and type IV collagen[7] and may be the causative agent in pulmonary emphysema[2] and rheumatoid arthritis. Modulation of the activity of HLE may lead to new and effective ways of alleviating these diseases.[8–10] This report describes the results of some *in vitro* studies involving the inhibition of HLE by a new class of inhibitors derived from hydantoin.

MATERIALS AND METHODS

Materials

Compound **1** was synthesized starting with L-valine as shown[11] in Scheme I. Compounds **2–4** were prepared as described in the literature.[12,13] Human leukocyte elastase and human leukocyte cathepsin G were purchased from Elastin Products Company and Athens Research and Technology Company, respectively. Chymotrypsin, methoxysuccinyl-ala-ala-ala-pro *p*-nitroanilide, succinyl ala-ala-pro-phe *p*-nitroanilide and *N*-Benzoyl-L-tyrosine ethyl ester were purchased from Sigma Chemical Co., St. Louis. The infrared and nmr spectra were recorded on a Perkin-Elmer infrared spectrophotometer and a Varian XL-300 spectrometer, respectively. A Gilford uv/vis spectrophotometer was used in the enzyme assays and inhibition studies. A Jasco DIP-360 automatic digital polarimeter was used to determine optical rotations.

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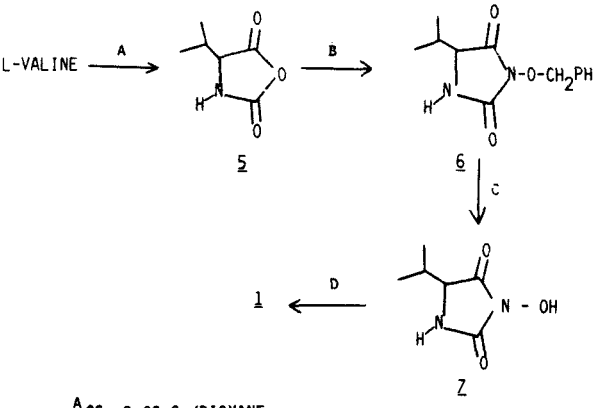


SCHEME I

L-Valine-N-carboxyanhydride (5). Trichloromethyl chloroformate (3.11 ml; 25.2 mmol) was added to a solution of L-valine (5.0 g; 43 mmol) in 200 ml anhydrous tetrahydrofuran kept at 40°C. The reaction was complete when all the amino acid had gone into solution (4 h). The solvent was removed in vacuo and the residue was treated with hexane to induce crystal formation. The crystals of the *N*-carboxyanhydride were collected and dried in a vacuum desiccator overnight (5.2 g; 85% yield), mp 68–71°C. IR (Nujol): 1740 cm^{-1} (C=O). NMR (DMSO-d_6): δ 0.84 (dd, 6H), 2.0 (m, 1H), 4.30 (d, 1H), 9.05 (br s, 1H).

L-3-Isopropyl-l-benzyloxyhydantoin (6). 5.2 g (40 mmol) of the *N*-carboxyanhydride in 150 ml anhydrous ethyl ether was added dropwise to a solution of benzyloxyamine (9.68 g; 80 mmol) in anhydrous ethyl ether (100 ml) and hexane (20 ml) at 0°C. The mixture was allowed to stir for 1 week at room temperature. The gelatinous precipitate that formed was filtered and washed with ethyl ether. The organic solvent was removed *in vacuo* and the residue was dried in a vacuum desiccator yielding crystals of compounds **6** (3.0 g; 33% yield), mp 76–77°C. IR (Nujol): 1750 cm^{-1}. NMR (Acetone-d_6): δ0.90 (dd, 6H), 2.0 (m, 1H), 3.72 (d, 1H), 5.0 (q, 2H), 7.40 (m, 5H).

L-3-Isopropyl-l-hydroxyhydantoin (7). 3.0 g (12.1 mmol) of compound **6** was dissolved in 100 ml tetrahydrofuran and hydrogenated (0.5 g 10% Pd-C/15 atm) for 20 min. Celite was added and the mixture was filtered. Evaporation of the solvent in vacuo left a solid residue (1.72 g; 90% yield), mp 150°C (dec.). IR (Nujol): 3230 (br OH), 1730 cm^{-1}. NMR (DMSO-d_6): δ0.89 (dd, 6H), 2.0 (M, 1H), 3.95 (d, 1H), 8.22 (s, 1H), 10.43 (s, 1H).

L-3-Isopropyl-l-(trans-styrylsulfonyloxy) hydantoin (1). *Trans*-styryl sulfonyl chloride (2.3 g; 11.4 mmol) was added to a solution of compound **7** (1.5 g; 9.5 mmol)

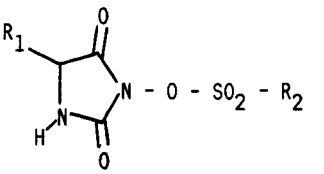
and pyridine (1.12 g; 14.2 mmol) in 10 ml toluene. The reaction mixture was stirred overnight at room temperature. Ethyl acetate (100 ml) was added, followed by dilute HCl (20 ml). The aqueous layer was extracted once more with ethyl acetate and the combined organic extracts were dried over anhydrous sodium sulfate. The solvent was removed in vacuo and the residue was recrystallized from ethyl acetate/hexane, mp 134–5°C (2.53 g; 82% yield). IR (Nujol): 1740 cm^{-1}. NMR ($CDCl_3$): δ0.92 (dd, 6H), 2.17 (m, 1H), 3.93 (d, 1H), 6.40 (s, 1H), 6.88 (d, 1H), 7.42 (m, 5H), 7.65 (d, 1H). **1** C, 51.85; H, 4.94; N, 8.64% (Theory) C, 51.66; H, 5.10; N, 8.56% (Found). **2** C, 46.48; H, 4.23; N, 9.86%. C, 46.24; H, 4.36; N, 9.70. **3** C, 56.67; H 4.44; N, 7.78%. C, 56.75; H, 4.64; N, 7.60%. **4** C, 58.06; H, 4.30; N, 7.53%. C, 58.20; H, 4.48; N, 7.75%. **5** C, 50.35; H, 6.29; N, 9.79%. C, 50.65; H, 6.51; N, 9.60%. **6** C, 62.90; H, 6.45; N, 11.29. C, 73.15; H, 6.32; N, 11.47%. **7** C, 45.57; H, 6.33; N, 17.72%. C, 45.77; H, 6.10; N, 17.50%.

Enzyme Assays and Inhibition Studies

Human leukocyte elastase (HLE) was assayed by mixing 10 μl of a 2.23 $\times$ 10^{-5} *M* enzyme solution (in 0.05 *M* sodium acetate buffer, pH 5.5), 10 μl DMSO and 980 μl of HEPES buffer, pH 7.2, in a thermostated test tube. A 100 μl aliquot was transferred to a thermostated cuvette containing 880 μl HEPES buffer and 20 μl of a 3.06 $\times$ 10^{-3} *M* solution of methoxysuccinyl ala-ala-pro-val *p*-nitroanilide and the change in absorbance was monitored at 410 nm for 2 min.

In a typical inhibition run, 10 μl of a 2.23 $\times$ 10^{-5} *M* enzyme solution was added to 880 μl HEPES buffer and 10 μl of inhibitor solution (2.23 $\times$ 10^{-4} *M* in DMSO) to initiate the inactivation. The residual enzymatic activity was determined by withdrawing aliquots (100 μl) at different time intervals and transferring them to a cuvette containing substrate (20 μl of a 3.06 $\times$ 10^{-3} *M* solution) and 880 μl HEPES buffer. After a 30 s incubation period, the absorbance was monitored at 410 nm for 2 min. The pseudo-first-order inactivation rate constants (k_{obsd}) were obtained from plots of $\ln(v_t/v_o)$ *vs* t and the apparent second-order inactivation rate constants $k_{obsd}/[I]$ $M^{-1}s^{-1}$ are listed in Table 1. These are the average of two determinations.

TABLE I
Hydantoin Derivatives



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Compound	R_1	R_2	$k_{OBSD}/[I]\,M^{-1}S^{-1}$
1	L-$(CH_3)_2$CH-	*trans*-Styryl	230
2	DL-Benzyl	Methyl	183
3	DL-Benzyl	*p*-Tolyl	118
4	DL-Benzyl	*trans*-Styryl	129

A similar procedure was used in evaluating the inhibitory activity of the compounds toward cathepsin G. Chymotrypsin was assayed as described previously.[14]

RESULTS AND DISCUSSION

Incubation of hydantoin **2** with human leukocyte elastase (HLE) resulted in a time-dependent and stoichiometric inactivation of the enzyme (Figures 1 and 2). Compounds **1**, **3** and **4** yielded similar plots. The $k_{obsd}/[I]$ values[15] for these compounds are

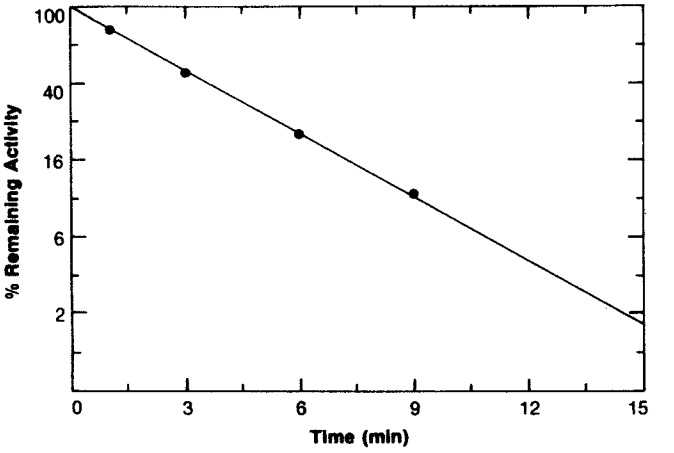

FIGURE 1 Kinetics of inactivation of human leukocyte elastase (HLE) by compound **2**. HLE (223 n*M*) was incubated with **2** (22.3 μM) in 0.1 *M* HEPES buffer, pH 7.2, and 1% DMSO. Aliquots were removed periodically and assayed for catalytic activity using methoxysuccinyl ala-ala-pro-val *p*-nitroanilide.

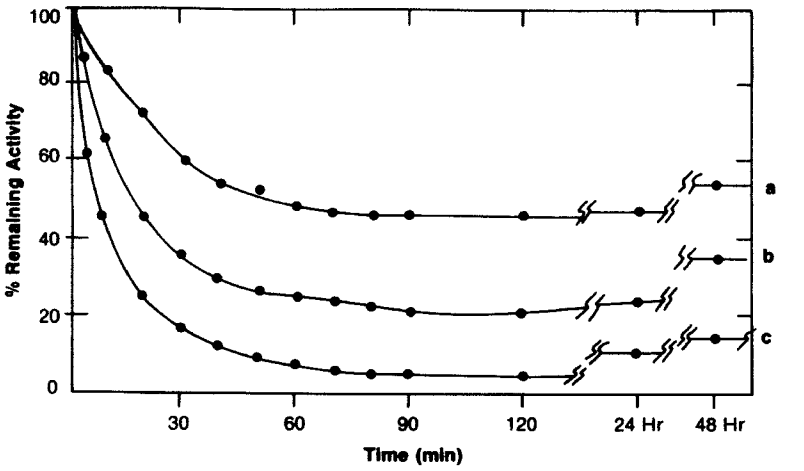

FIGURE 2 Time-dependent loss of enzymatic activity. Inhibitor (compound **2**) to enzyme ratios were: *a* (10:1), *b* (20:1) and *c* (30:1), 0.1 *M* HEPES buffer, pH 7.2.

listed in Table I. The enzyme-inhibitor adduct is fairly stable to reactivation as evidenced by the fact that fully-inactivated enzyme regained 12% (24 h) and 16% (48 h) of its activity when allowed to stand in HEPES buffer, pH 7.5. In a separate experiment, when fully-inactivated enzyme was treated with buffered hydroxylamine, partial and rapid reactivation of the enzyme was observed (Figure 3). This observation strongly suggests the formation of labile acyl or other linkages between the inhibitor and the enzyme. Dialysis of fully-inactivatd enzyme over a 24 h period against 0.1 *M* HEPES buffer, pH 7.5, did not lead to any recovery of enzymatic activity over that observed above, suggesting that the inactivation is irreversible. These inhibitors interact with the active site of the enzyme, since the rate of inactivation decreased in the presence of added substrate (Figure 4, $k_{obsd}/[I] = 51\,M^{-1}s^{-1}$). The results described herein indicate that the inactivation is irreversible and involves the covalent modification of one or more active site residues (presumably Ser-195 and/or His-57). Cathepsin G and chymotrypsin are also inhibited by this class. Compound **3**, for example, produced 83% and 73% inhibition with chymotrypsin and cathepsin G respectively after incubating for 10 min at an inhibitor to enzyme ratio of 200.

An assessment of the relative stability of this class of compounds in 0.1 *M* HEPES buffer, pH 7.5, was made by using compound **2**. The structural integrity of compound **2** remained unaffected after 24 h in buffer, as demonstrated by high-pressure liquid chromatography (Chromagabond MC 18 15 cm × 4.6 mm column, 80% CH_3CN/H_2O, 10 ml/min flow, 275 nm).

Mechanistic studies related to the Lossen rearrangement[16] and earlier observations made by us[17-18] strongly suggested that derivatives of hydantoin represented by **I** might function as inhibitors of proteolytic enzymes. The results described in this communication bear out the validity of our approach. It is clear from Table I that

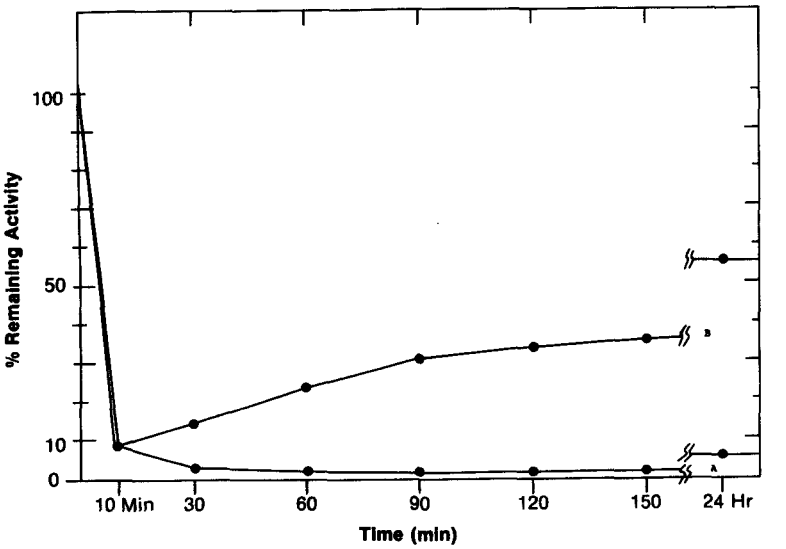

FIGURE 3 Recovery of enzymatic activity. Conditions: *A*: [HLE], 272 n*M*; [Inhibitor **2**], 27.2 μM in 0.1 *M* HEPES buffer, pH 7.2. *B*: [HLE], 272 n*M*; [Inhibitor **2**], 27.2 μM; [NH_2OH], 45.4 m*M* in 0.1 *M* HEPES buffer, pH 7.2.

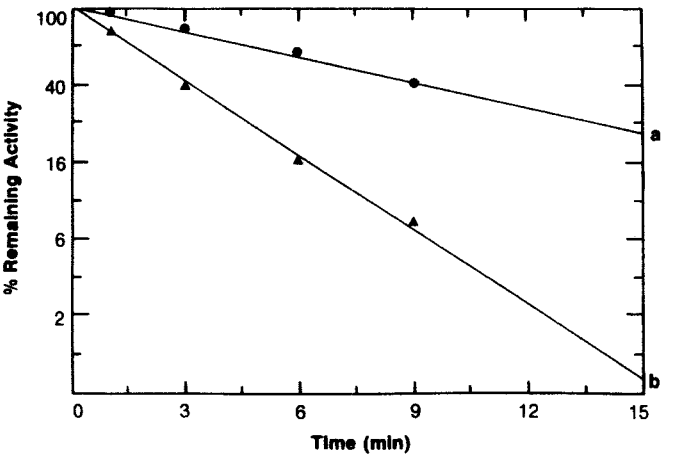

FIGURE 4. Protection of HLE by substrate. Conditions: *a*: [HLE], 300 n*M*; [Inhibitor **2**], 30.0 μM; [Methoxysuccinyl ala-ala-pro- val *p*-nitroanilide], 0.85 m*M*. *b*: [HLE], 300 n*M*; [Inhibitor *2*], 30.0 μM, 0.1 *M* HEPES buffer, pH 7.2.

hydantoin derivatives are efficient inhibitors of human leukocyte elastase and other proteolytic enzymes. Manipulation of the R_1 and R_2 groups should lead to the development of inhibitors of greater potency and specificity. Furthermore, the high stability of this class of inhibitors renders their utilization as enzyme probes and potential therapeutic agents highly advantageous.[8] The mechanism by which these compounds exert their inhibitory activity is currently under investigation.

Acknowledgement

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